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METABOLISM OF SULFUR AMINO ACIDS IN MYTILUS EDULIS AND RANGIA CUNEATA [1]

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Sulfur amino acids are important metabolites in living systems. Their metabolism is well known in mammals—the reactions involved in their breakdown are known and so are the end products formed. In invertebrates, however, less is known about the metabolism of sulfur amino acids. It is known that some marine invertebrates contain in their tissues large amounts of taurine, (Kelley, 1904; Henze, 1905; Mendel, 1904; Kossel and Edlbacher, 1915; Okuda, 1920; Ackermann *et al.*, 1924) but not much is known of its origin or its role in the animal. Recently, several papers have been published in which the probable origin of taurine is mentioned. In one paper, Shibuya and Shunji (1957) reported that hypotaurine, a precursor of taurine, was present in *Septifer virgatus;* Ouchi (1959) claimed that hypotaurine was present in numerous species of molluscs. Hypotaurine, first found in the rat by Awapara (1953), is formed from cysteine sulfinic acid and converted to taurine as demonstrated by Awapara and Wingo (1953). Taurine, then, could probably be formed in invertebrates by the same sequence of reactions as in the rat. Cotty *et al.* (1958) studied the metabolism of sulfur amino acids in *Musca domestica* and concluded that methionine serves as a precursor of taurine.

In a recent paper, we reported that taurine is present in all marine invertebrates studied, but was absent or not detectable in fresh water and terrestrial molluscs (Simpson, Allen and Awapara, 1959). This comparative study did not tell us whether sulfur amino acids are metabolized differently by marine molluscs and fresh-water molluscs. The end products of sulfur metabolism could be different; we needed to know more about the intermediate steps. We have performed a number of experiments on *Mytilus edulis,* which is known to have large amounts of taurine, and on *Rangia cuneata* which has none detectable by paper chromatography. The results of these experiments show that both can convert methionine to cysteine, probably in the same manner as mammals do, and that cysteine can be oxidized to various products some of which can be decarboxylated to give taurine.

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[2] The data reported here is part of the Ph.D. thesis of Kenneth Allen.

Materials and Methods

Animals

M. edulis was obtained from the Marine Biological Laboratory, Woods Hole. The animals were put in sea water cooled to 5° C. and kept there until they were used. *R. cuneata* was collected from the San Jacinto River, Harris County, Texas. They were kept in large aerated aquaria.

Chemicals

S^{35}-methionine, S^{35}-cystine and S^{35}-sodium sulfite were obtained from Abbott Laboratories, Oak Ridge. They were chromatographically pure. S^{35}-taurine was prepared from S^{35}-sulfite and β-bromoethylamine hydrobromide according to the method of Cortese (1943). The crude taurine was purified by chromatography on a column of Dowex 50 in the H^+ form. The final product was chromatographically pure. Cystine was reduced to cysteine according to the method of Lucas and Beveridge (1940).

Administration of compounds

Solutions of S^{35}-methionine, S^{35}-cysteine and S^{35}-taurine were prepared and administered to both *M. edulis* and *R. cuneata* in the following manner: the shell of *M. edulis* was opened and kept open with wound spreaders. The foot was located and 1 ml. of the solution carefully injected into the foot; a 1-ml. syringe with a 22-gauge needle was used. This method was suggested by Dr. T. W. Potts, of the University of Birmingham, England. *R. cuneata* was injected through a hole cut carefully in the shell; the needle was inserted through the mantle and gill into the fleshy part of the viscera. After the injection the animals were returned to aquaria containing water of the appropriate salinity and temperature.

Extraction and fractionation of amino acids

Extractions were carried out by the method of Awapara (1948). The extracts were used in some instances for paper chromatography and in others for isolation of intermediates. Most of the time the extracts were fractioned into various groups, using ion exchange resins according to the scheme shown in Table I. According to this scheme the extracts containing one gram of tissue per cc. were first put through a column (10 cm. × 1 cm.) of Dowex 50 in the H^+ form (200–400 mesh). The column was washed with distilled water until the effluent became neutral. All the anions and non-polar compounds were washed through by this procedure, along with taurine, cysteic acid and cysteine sulfinic acid. All other amino acids were eluted from the resin bed with 25 ml. of 2 N NH_4OH; the acid effluent (A) and the NH_4OH eluate (B) were evaporated to dryness; the residues redissolved in a small amount of water. Fraction A, which contained taurine, cysteic acid, cysteine sulfinic acid and sulfate, was fractionated using a strong anion exchange resin—Dowex 2 in the OH^- form. The solution was put through a small column of the resin (5 cm. × 1 cm.) and washed with 50 ml. of boiled and cooled distilled water. Elution was carried out in two stages: (1) with 10 ml. of 2 N acetic acid to remove taurine, and (2) with 10 ml. of 2 N HCl to remove

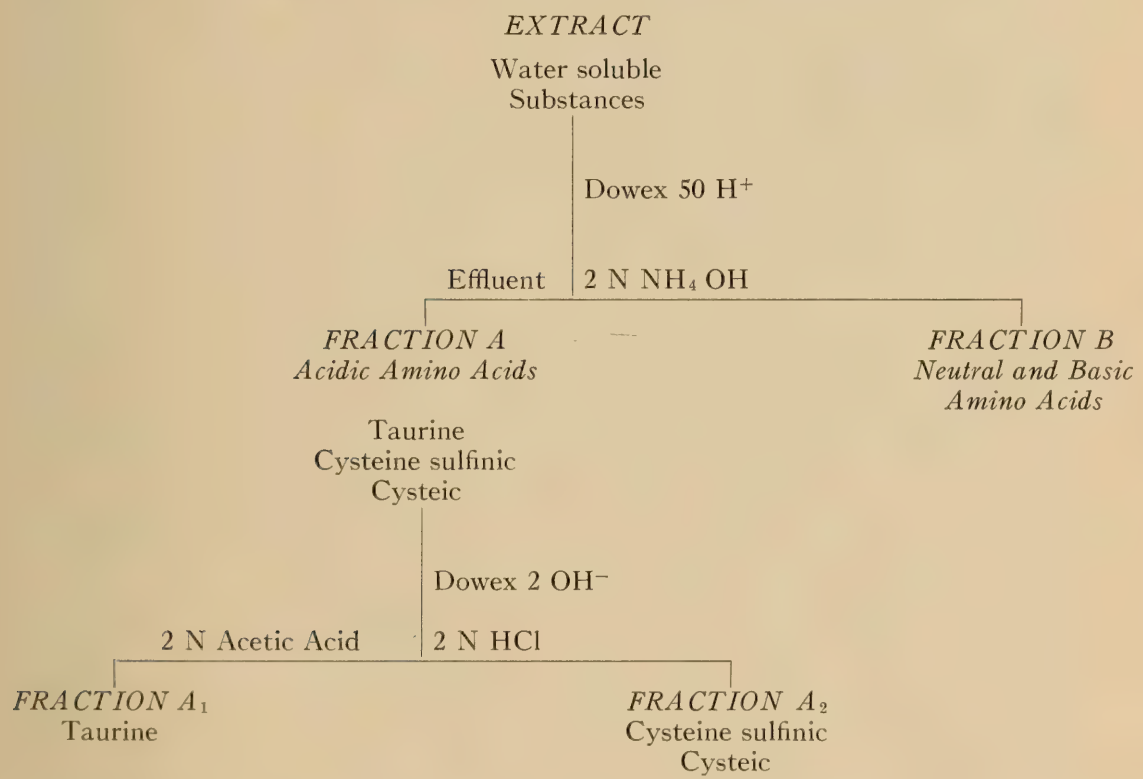
cysteic and cysteine sulfinic acids. Fractions A_1 and A_2 were evaporated to dryness; the residues were dissolved in small amounts of water and the evaporation repeated to drive off most of the acid. Finally the residues were taken up in small portions of water and stored for further work.

Identification of products

Intermediates and final products were identified by several methods: (a) The various fractions were chromatographed on paper; radioautographs were made of these papers using x-ray film. After a three- to five-week exposure the papers

TABLE I

Fractionation of extracts by means of ion exchange resins



were developed with ninhydrin. The ninhydrin spots were matched against the blackened areas on the film. Tentative identifications were thus made on the basis of R_f values. (b) Some of the fractions were chromatographed on paper strips and the strips analyzed for radioactivity in a continuous strip counter. The position of the peaks was used as criterion for identification. Also, chromatography on strips was carried out using the various unknown fractions but mixed with known substances suspected to be present in the fractions. A perfect overlap between the peaks and the ninhydrin spots on the strip was used as criterion of identity. (c) When sufficient evidence was available for the existence of an intermediate, it was isolated, purified and its radioactivity determined. If the amounts present were insufficient for isolation, carrier was added, then isolated, purified by recrystallization or precipitation and its radioactivity determined. When a compound was isolated without carrier, other criteria were used such as infrared spectrum.

Results

1) *Formation of cystathionine*

Both *M. edulis* and *R. cuneata* were given a solution of S^{35}-methionine (approximately 500,000 counts). After 24 hours the organisms were extracted and the extracts fractionated. Fraction B in each case was chromatographed on paper

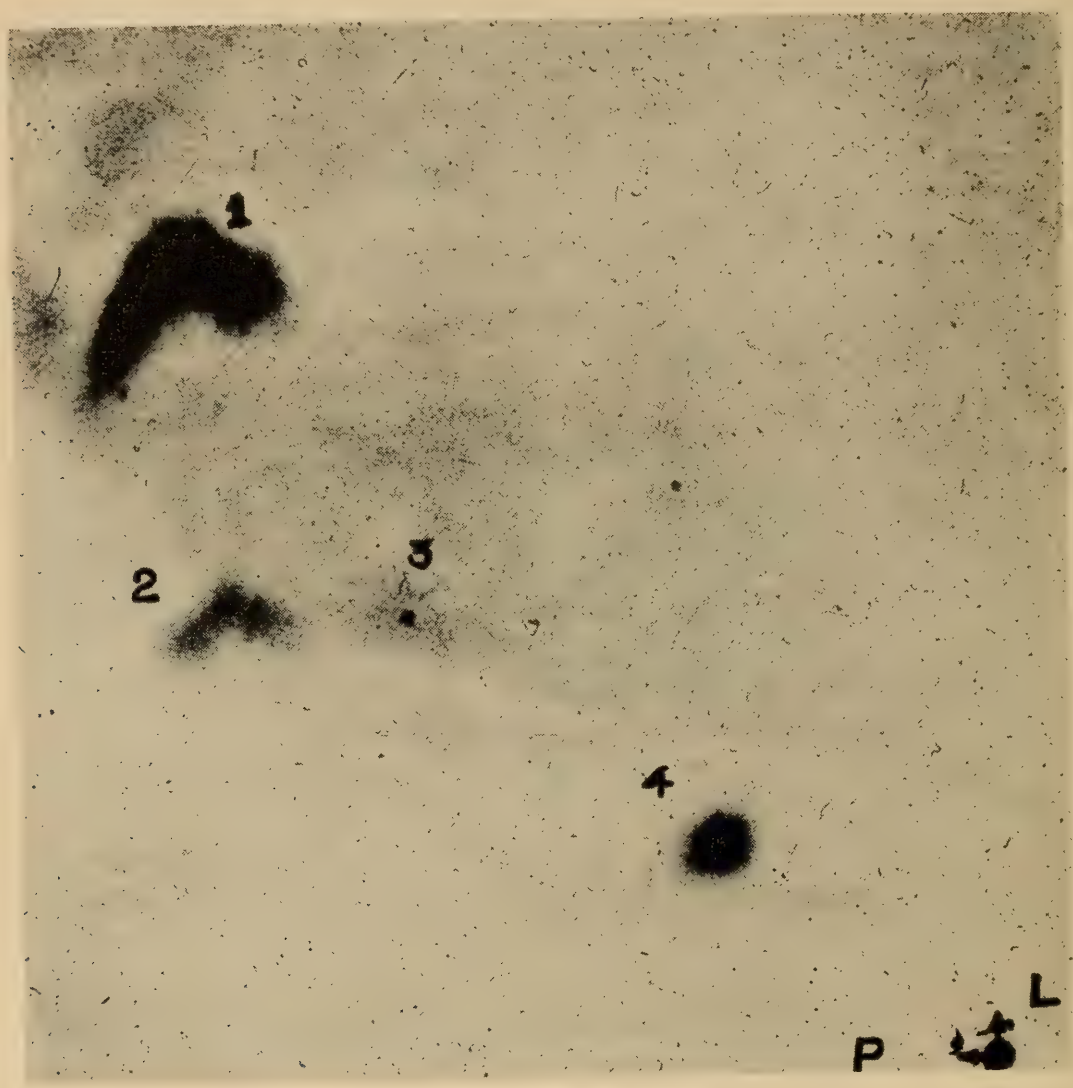


Figure 1. Radioautograph of fraction B from *M. edulis* after the administration of S^{35}-methionine. Solvents: phenol (P) and lutidine (L). Methionine (1), methionine sulfone ? (2), hypotaurine (3) and cystathionine (4).

according to the method used by Simpson, Allen and Awapara (1959), using phenol-water in one direction and lutidine-water in the second direction. Radioautographs of these chromatograms were made. The chromatograms from *R. cuneata* had no cystathionine. We cannot exclude its formation, for it could easily be a transient intermediate in this animal. It is very likely that it is formed, as we shall see in discussing formation of cystine. In Figure 1 is shown a radioautogram from *M. edulis*. Spot 4 is cystathionine and spot 3 is hypotaurine. Both of these

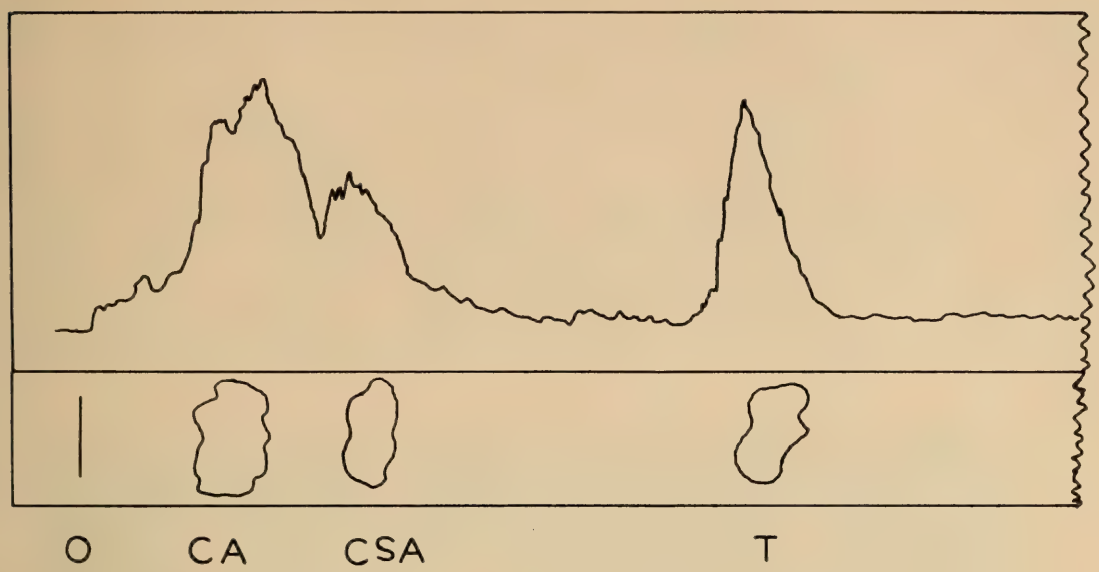


FIGURE 2. Strip chromatogram (lower) and radioactivity distribution (upper) from the acid fractions of *R. cuneata*. Solvent: phenol-water. Cysteic acid (CA), cysteine sulfinic acid (CSA) and taurine (T).

intermediates were recognized by their R_f values (cystathionine 0.27 in 72% phenol and 0.20 in 65% lutidine; hypotaurine 0.62 in 72% phenol and 0.42 in 65% lutidine). Spot 1 is methionine and spot 2, probably methionine sulfone.

2) Formation of cystine

Fraction B, from part 1, was used to identify cystine or cysteine. Neither of these amino acids can be chromatographed satisfactorily. Cystine is very insoluble and lends itself to isolation. In both instances unlabelled cystine hydrochloride was added to the fractions. Then hydrogen peroxide was added to oxidize cysteine to cystine. Cystine was precipitated from the solution by carefully adjusting the pH to 7. The crystals were filtered, washed, redissolved in dilute HCl and

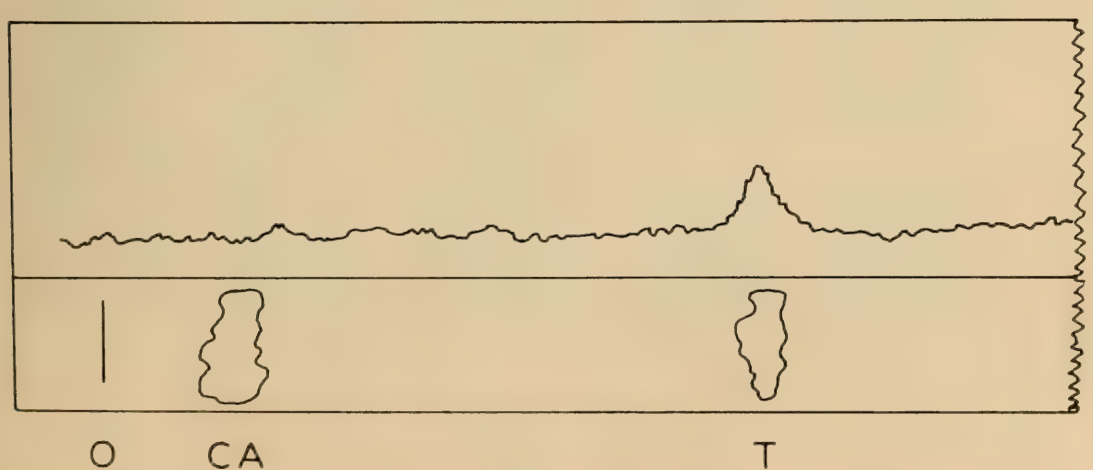


FIGURE 3. Strip chromatogram and radioactivity distribution from the acid fraction of *M. edulis*. Cysteic acid (CA) and taurine (T).

reprecipitated by the same procedure. The washed and dried crystals were used for determining radioactivity.

M. edulis	68 counts/minute/mg.
R. cuneata	120 counts/minute/mg.

Cystine or cysteine is formed in both organisms. Cysteine oxidizes readily to cystine but if one assumes that transulfuration takes place in these animals, then cystathionine and cysteine are the intermediates. Unfortunately cystathionine was not detected in *R. cuneata*. If it is not formed we must postulate another mechanism for the formation of cysteine and no other mechanism is known at this time.

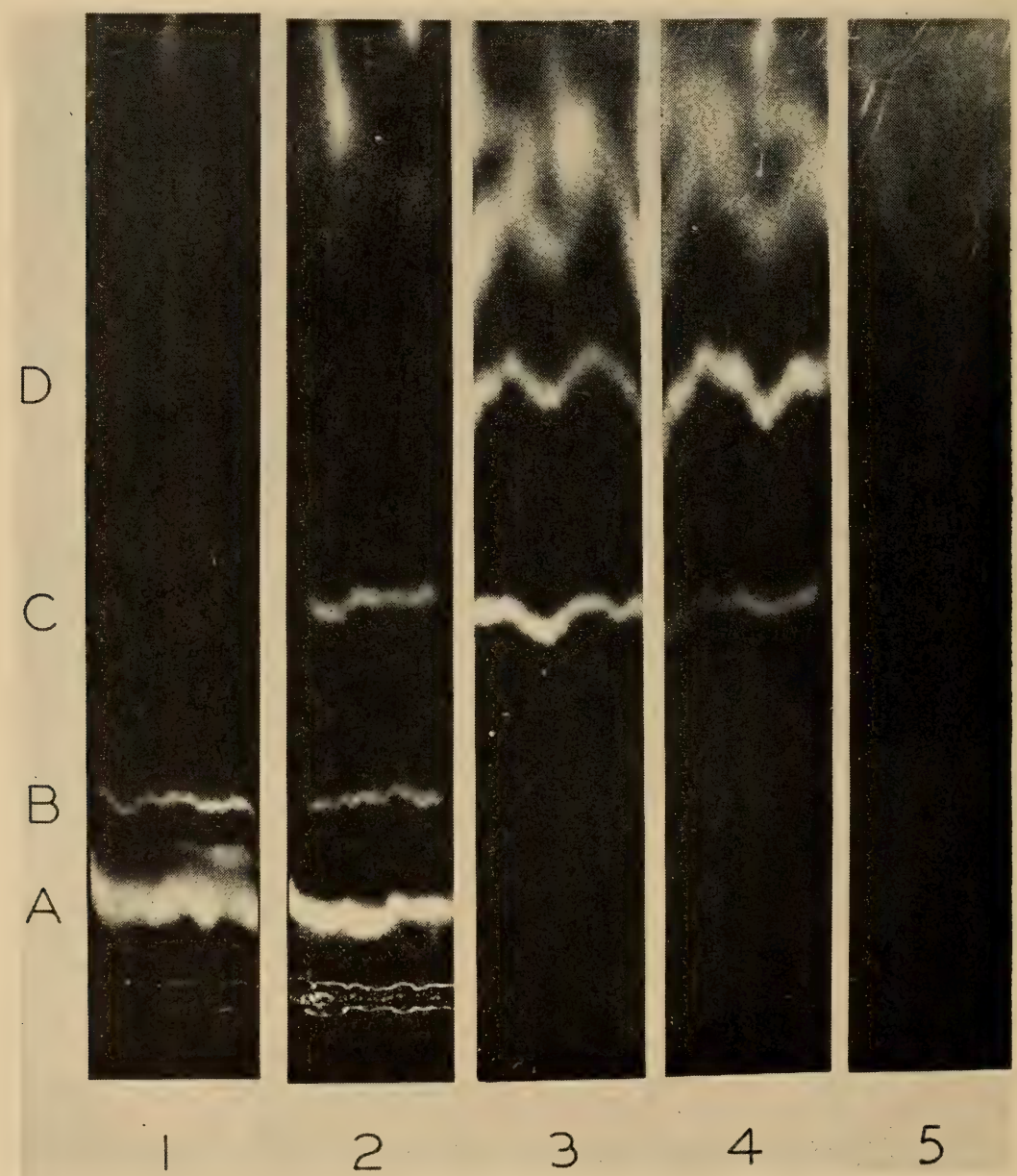


FIGURE 4. Radioautographs of the acid fraction of *R. cuneata* after the administration of methionine. (1) After 1 hour, (2) after 5 hours, (3) after 10 hours, (4) after 15 hours and (5) after 24 hours. (A) cysteine sulfinic acid, (B) cysteic acid, (C) taurine, (D) unknown.

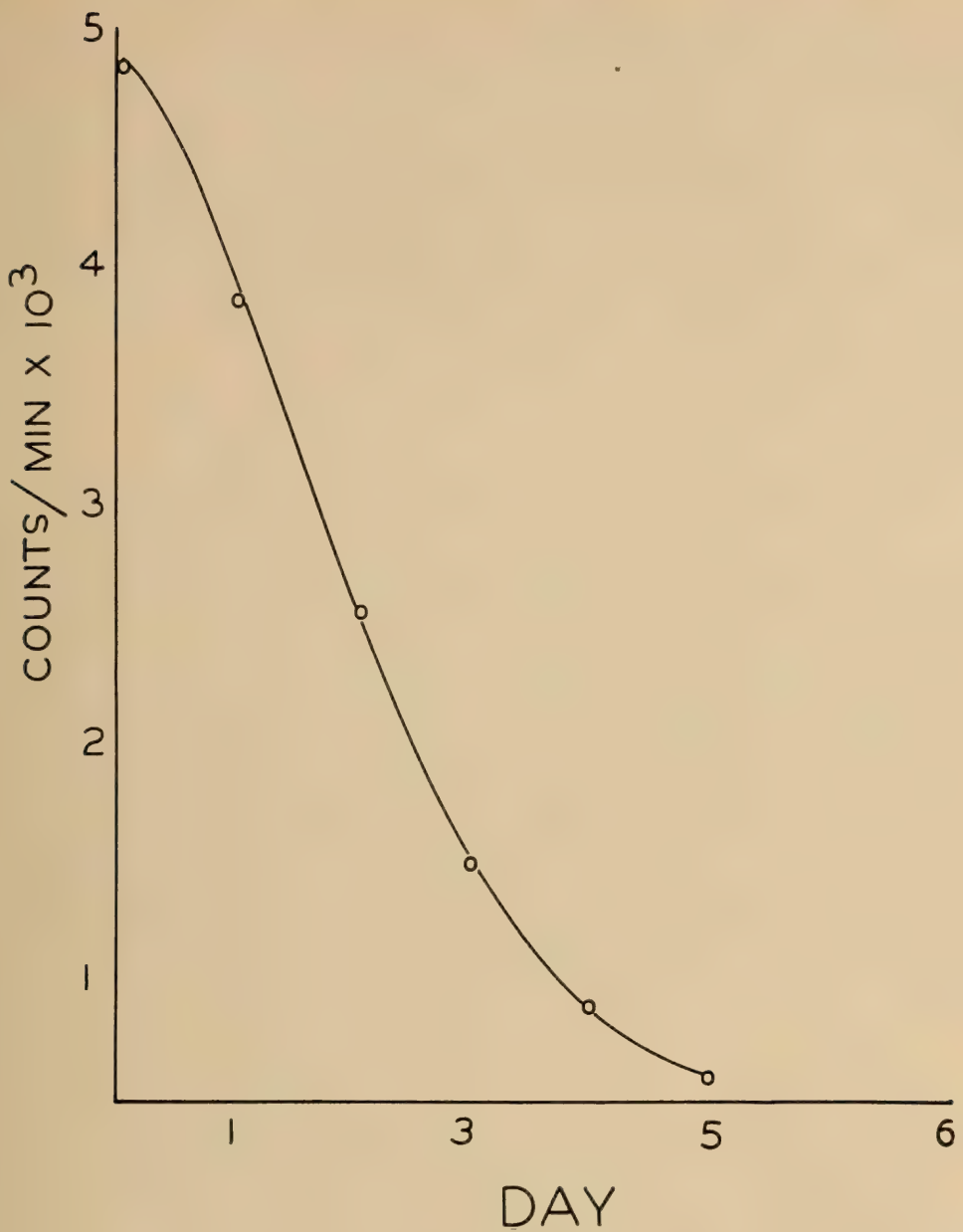


FIGURE 5. Disposition of administered S^{35}-taurine by *R. cuneata.*

3) *Oxidation products of cysteine*

Pooled fractions A_1 and A_2 from both organisms were mixed with cysteic acid, cysteine sulfinic acid and taurine, then chromatographed using 72% phenol. The strips were analyzed with a continuous strip counter, then developed with ninhydrin. In Figures 2 and 3 are shown the results obtained for *R. cuneata* and *M. edulis.* The latter had only one radioactive spot: taurine. Cysteic acid was formed in *R. cuneata* in relatively high amounts. A second experiment was performed on *R. cuneata* to find out the time needed for the formation of these intermediates. S^{35}-methionine was injected into several organisms; extracts were made at the end of 1, 5, 10, 15 and 24 hours. The extracts were fractionated and fraction A of each one chromatographed on strips. Radioautograms were made and they are shown in Figure 4. Taurine was formed only after cysteine sulfinic acid and

cysteic acid were formed; it took 5 hours to form sufficient taurine to be detected. At the end of 10 hours two S^{35}-labelled compounds appeared. Compound D has an R_f value identical to that of taurocyamine (.64 in phenol) but gave a negative Sakaguchi test on paper chromatograms. At the end of 24 hours most of the end products have disappeared from the animal. Taurine is formed in *R. cuneata* but not held. This is better shown in Figure 5. In this figure is shown the rate at which injected S^{35}-taurine is excreted by *R. cuneata*. One could argue that the conditions were abnormal and that the animal ejects an exogenous substance; this is not the case as seen in Figure 5; taurine is endogenously formed and it is also rapidly excreted.

4) *Formation of sulfate and taurine*

Both sulfate and taurine are formed by *M. edulis* and *R. cuneata*. Sulfate was detected by precipitation as $BaSO_4$ with added carrier. In both instances the $BaSO_4$ was radioactive (35 cts./min. and 85 cts./min.). Although the counts were low, they were significant. The $BaSO_4$ was ignited to insure destruction of any organic sulfur compounds.

Taurine was detected in *R. cuneata* as already shown; *M. edulis* is known to contain very large amounts of taurine (as much as 4 per cent of wet weight) and to demonstrate its formation from administered precursors would be difficult. Nevertheless we succeeded in isolating S^{35}-labelled taurine from *M. edulis* after the administration of S^{35}-methionine. The isolated taurine was recrystallized several times and its radioactivity determined. The specific activity was 27 counts/min./mg. With such low activity the evidence for taurine formation was somewhat weak. More supporting evidence was obtained by isotope dilution. To the isolated taurine we added an equal amount of pure taurine; the mixture

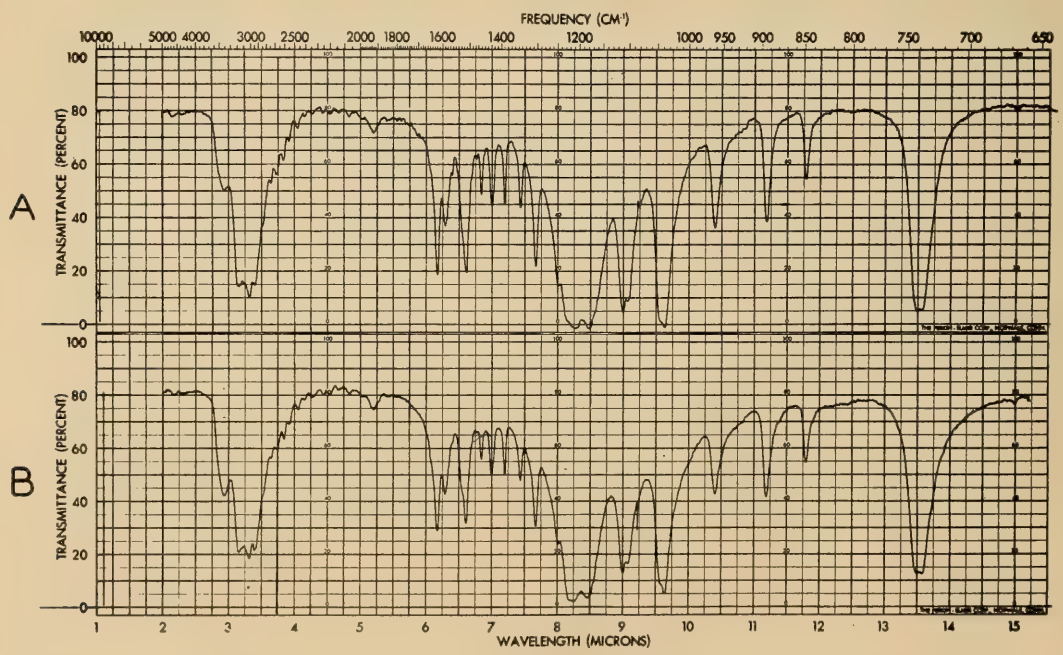


FIGURE 6. Infrared absorption spectra of (A) known taurine and (B) S^{35}-taurine isolated from *M. edulis* after the administration of S^{35}-methionine.

was recrystallized and the radioactivity of the pure crystals determined:

Isolated taurine	6 mg.	26 cts./min./mg.
Mixture	12 mg.	12 cts./min./mg.

The infrared absorption spectra [3] of both pure taurine and the isolated taurine were determined and shown in Figure 6. It appears that *M. edulis* forms taurine slowly but retains it. The high concentration of endogenous taurine in this animal makes it very difficult to establish the rate at which taurine is formed.

Discussion

The metabolism of sulfur amino acids in the two molluscs studied is not different, at least qualitatively, from the metabolism in mammals. The oxidation of cysteine in both, mammals and molluscs (those studied), gives rise to cysteine sulfinic acid and eventually to taurine and sulfate (Awapara, 1956). The most significant difference observed in the two animals studied is the rate at which taurine is disposed of. *M. edulis* keeps it by some unknown mechanism defying a concentration gradient. *R. cuneata* can produce taurine but cannot hold it. If we extrapolate these results, the absence of taurine in all the fresh-water animals (Simpson, Allen and Awapara, 1959) could be explained on the basis of rapid excretion, not lack of formation. The method of formation of taurine and sulfate in both organisms again appears similar to the method of formation in mammals. Cysteine sulfinic acid is either decarboxylated to hypotaurine or forms sulfate after transamination, splitting of SO_2, and oxidation. The absence of hypotaurine in *R. cuneata* and the presence of large amounts of cysteic acid is indicative of a pathway in which cysteine sulfinic acid is oxidized first to cysteic acid and this decarboxylated to taurine. In *M. edulis* hypotaurine was present but cysteic acid was not detected. If there are any differences in the metabolism of sulfur amino acids in the molluscs studied, the differences are in the intermediates and in the disposition of the end products.

The role of taurine in marine molluscs remains unknown. The only suggestion made is that it serves as an osmoregulator.

Summary

1. The metabolism of sulfur-bearing amino acids was investigated in *R. cuneata* and *M. edulis, in vivo*.

2. Injected S^{35}-methionine gives rise to cysteine/cystine in both animals. Cystathionine was detected in *M. edulis*, but not in *R. cuneata* though it is probably formed. A demethylation and transulfuration mechanism is postulated for this conversion.

3. Both *R. cuneata* and *M. edulis* form sulfate from injected methionine. Also, both form taurine but *R. cuneata* does not accumulate it whereas *M. edulis* accumulates it in large amounts.

4. Taurine is probably formed by different reactions in these species. In *R. cuneata* it is probably formed by decarboxylation of cysteic acid, whereas in *M. edulis* it is formed by oxidation of hypotaurine.

[3] We are indebted to Dr. T. Patton from the M. D. Anderson Hospital for this determination.

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